Protein and Nitrate Content of *Lemna* Sp. as a Function of Developmental Stage and Incubation Temperature[1]

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ABSTRACT

Lemna protein per frond and per root increases with developmental stage until plants are at least two generations old. Protein per frond, per root, and per unit dry weight is greater in plants grown at 23.9 C than at 18.3 C. More protein is found in fronds than in roots, and more nitrate occurs in roots than in fronds. Nitrate per root increases with developmental stage and is higher (per root) in plants grown at 23.9 C than in those grown at 18.3 C. The distribution of generations within a growing population is constant for at least eight doubling times. Whether populations multiply slowly at 15.6 C or more rapidly at 23.9 C, fronds which have not yet produced progeny form 62% of the population; fronds which are one generation old form 24% of the population; and fronds which are two generations old form 9% of the population.

These experiments were undertaken to evaluate the possibility of using duckweeds to remove nitrate from irrigation return water. Protein and nitrate were measured in fronds and roots at different developmental stages. The distribution of developmental stages in the growing population was also monitored. Results allow preliminary estimates of nitrate removal as a function of time after frond inoculation, but the great difference between protein content (per plant and per unit dry weight) of plants grown at 18.3 C and at 23.9 C implies that reliable models will require a large data base.

MATERIALS AND METHODS

Culturing of Plants. *Lemna* (species unknown)[2] was obtained from local ponds, sterilized by hypochlorite treatment (10), and grown in sterile Hoagland solution modified to contain 20 mg l^{-1} nitrogen (nitrate-N). This nitrate concentration was chosen because it is typical of agricultural return water in the area (8). The modification did not affect the pH of the incubation medium. All plant material used for the experiments was derived from a single progenitor frond and was presumably genetically identical. Culture vessels were Kimax crystallizing dishes containing 250 ml sterile medium and attached by parafilm to sterile, disposable Plexiglas covers. Dishes were incubated in growth chambers at 15.6 C, 18.3 C, or 23.9 C, under continuous irradiation from 14 metal halide lamps plus 12 self-ballasted mercury lamps with deluxe white phosphor which, together, provided 350 μE m^{-2} s^{-1} on the surface of the dish lids. Nutrient medium was changed daily by gently lifting plants into new dishes containing freshly sterilized medium. 'Frond' is used in this paper in the sense of Ashby *et al.* (1) and refers to a single leaf from which root and younger (or older) attached appendages have been dissected away. 'Plant' is a frond with its root and pocket containing developing fronds. 'Colony' is used in the sense of Datko *et al.* (4) and refers to a collection of plants which are physically bound as a group.

Protein, Nitrate, and Dry Weight Determinations. For protein assays, five replicate samples of 20 plants were harvested from each generation. A razor blade was used to separate fronds from roots. Upon harvesting, tissue was chilled, rinsed with deionized H$_2$O, and ground in a homogenizer, and the extract was frozen. The frozen samples were accumulated gradually over a period of several months. Accumulated extracts were washed with 5% trichloroacetic acid and assayed for soluble protein by the Lowry method (17).

For nitrate analysis, five replicate samples of nine plants were harvested from each generation. After separation of fronds from roots, tissue was chilled, rinsed with deionized water, ground in a homogenizer, and filtered through a Millipore HA filter (0.45-μm pore size), and the filtrate was frozen. Nitrate concentration was measured by the high pressure liquid chromatography method of Thayer and Huffaker (20).

Dry weight was determined on five replicate tissue samples, containing between 9 and 20 fronds or roots, after 24 h of incubation at 41 C.

Generation Study. Frond lineage was followed by marking fronds with small dots of colored ink (which appeared not to harm fronds or change growth rates) and periodically placing siblings into separate, marked culture vessels. The development is continuous; but, for convenience when a new frond emerged from a side pocket, it was called the 0 (daughter) stage, and it simultaneously caused its progenitor frond to reach the 1 (parent) stage. The numbers of fronds at each generational stage were recorded daily for approximately 2 weeks at 15.6 C, 18.3 C, and 23.9 C.

Numerical Methods. In analyzing the growth data (Figs. 9, 10) for each temperature, the conventional relationship, (ln total frond number) = A × time + B, was assumed. The regression coefficient A and the y-intercept B were calculated using a linear regression program. Among the different experiments, A varied with temperature and B with initial population size. For each point in Figures 9 and 10, the number of doubling times was then computed as (time × A)/ln 2, and log$_2$ (frond number) was computed as (frond number − B)/ln 2. This transformation had the effect of normalizing each time course to an initial value of one frond at time zero. In actual experiments, the initial total frond number was between 8 and 29, with the final population growing to well over 500 to 600 fronds. A two-way analysis of variance was used to determine whether the differences between the two incubation temperatures were significant. To test for monotonic increase of

[2] Cultures and preserved specimens of the locally collected *Lemna* are maintained in the laboratory of Dr. Allen W. Knight.

a variable θ with developmental stage (H_0:no difference among stages; H_a:$\theta[0] \leq \theta[1] \leq \theta[2]$), a nonparametric test, the Jonckheere-Terpstra test, was used (15).

RESULTS

Protein Content of Fronds and Roots. Protein content per frond increases with developmental age between the nonexpanded juvenile and second generation (grandparent) stages of development. The protein increase with age occurs in plants grown at both 18.3 C and 23.9 C (Fig. 1) and is statistically significant even when only the fully expanded fronds are considered (Table I). Average protein content is 1.7- to 3.1-fold higher in fronds grown at 23.9 C than in those grown at 18.3 C (Fig. 1). The change in protein
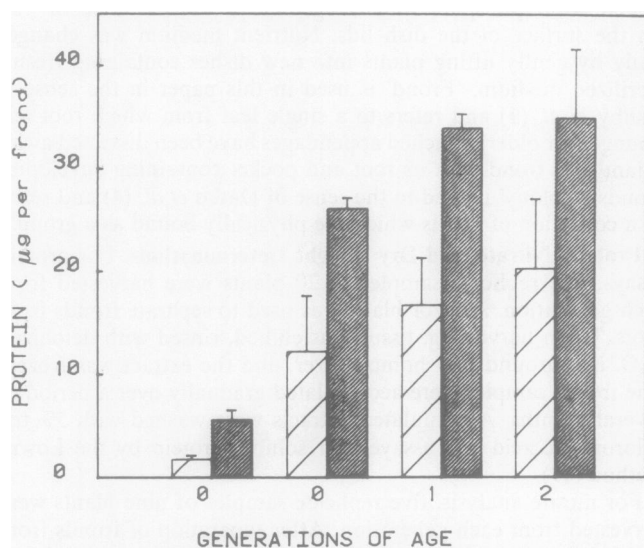

FIG. 1. Frond protein (μg) in fronds of different developmental stages (left to right): nonexpanded daughter fronds, expanded daughter fronds, parent fronds, and grandparent fronds. Light bars represent samples grown at 18.3 C; dark bars represent samples grown at 23.9 C. T-shaped lines indicate SD for each sample.

Table I. *Jonckheere-Terpstra Test for Increase with Generational Stage*

Variable	Incubation Temp	J Value	Significance Level
	C		
Protein per frond	18.3	41	*[a]
	23.9	53	**[b]
Protein per root	18.3	72	**
	23.9	73	**
Nitrate per frond	18.3	11	NS[c]
	23.9	−39	NS
Nitrate per root	18.3	75	**
	23.9	57	**
Dry weight per frond	18.3	51	**
	23.9	47	*
Dry weight per root	18.3	75	**
	23.9	63	**

[a] *, $0.01 < P < 0.02$.
[b] **, $P < 0.01$.
[c] NS, not significant.

Table II. *Analysis of Variance with Temperature*

Two-way analysis of variance with one degree of freedom for differences explained by temperature.

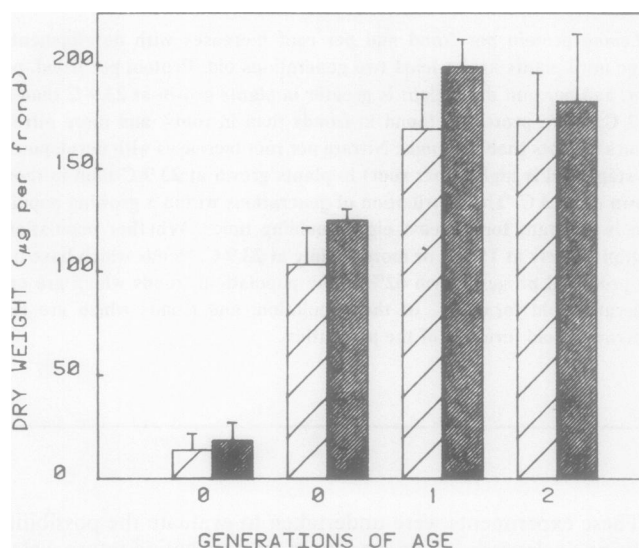| Variable	F Value	Degree of Freedom	Significance Level
Protein per frond	85.67	(1, 32)	***[a]
Protein per root	33.00	(1, 27)	**
Nitrate per frond	36.44	(1, 32)	**
Nitrate per root	73.94	(1, 24)	**
Dry weight per frond	9.07	(1, 32)	**
Dry weight per root	9.81	(1, 24)	**

[a] **, $P < 0.01$.

FIG. 2. Frond dry weight (μg) as a function of developmental stage and of temperature. Legend as for Figure 1.

Table III. *Protein and Nitrate-N as Percentage of Dry Weight*

Substance	Tissue Type	Incubation Temp	Generational Stage			
			0 (nonexpanded)	0 (expanded)	1	2
		C				
Protein	Fronds	18.3	13	12	10	12
		23.9	30	21	17	19
	Roots	18.3		11	8	8
		23.9		13	10	11
Nitrate	Fronds	18.3	0.1	0.07	0.05	0.05
		23.9	0.6	0.1	0.09	0.07
	Roots	18.3		0.4	0.6	0.8
		23.9		0.8	0.7	0.8

content with temperature is highly significant (Table II).

Inasmuch as frond dry weight may also increase with temperature and with developmental age (Fig. 2; Tables I, II), we calculated protein as percentage of dry weight for all treatments (Table III). The data in Table III were not suitable for statistical analysis, because dry weight and metabolite concentrations had to be determined on separate samples. In addition, the values for the nonexpanded juveniles are particularly in doubt, since dry weight increases rapidly within this stage. Nevertheless, it can be inferred that, at a given temperature, the percentage of protein remains constant throughout the frond's development. Percentage of pro-
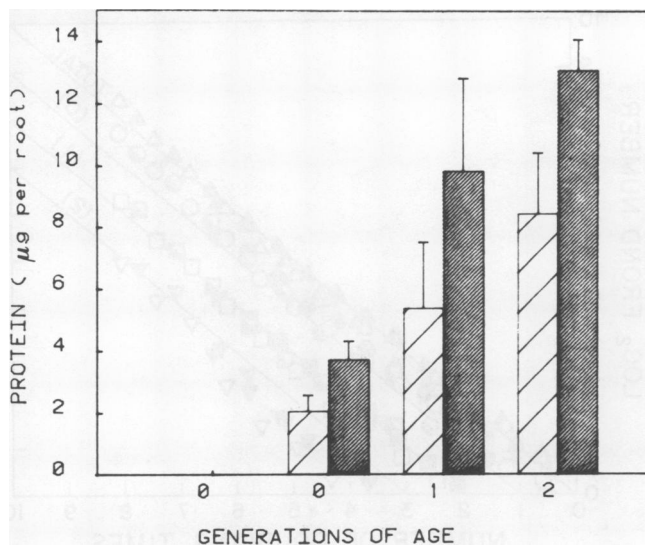
FIG. 3. Root protein (μg) as a function of developmental stage and of temperature. (Roots are barely detectable before full frond expansion.) Legend as for Figure 1.
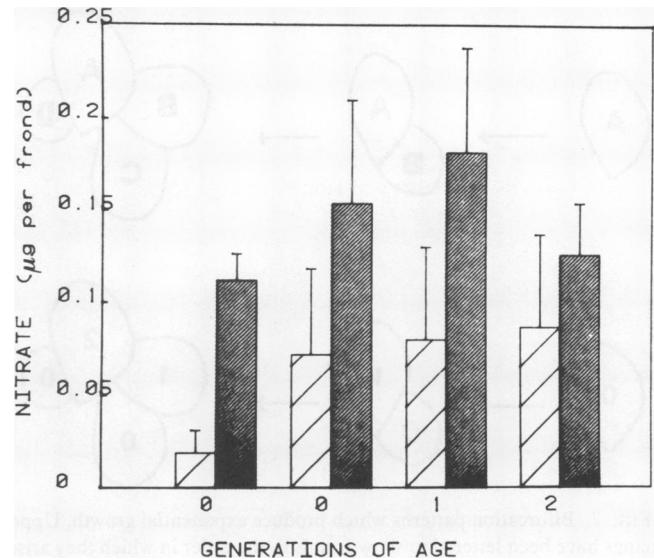

FIG. 5. Frond nitrate (μg) as a function of developmental stage and of temperature. Legend as for Figure 1.
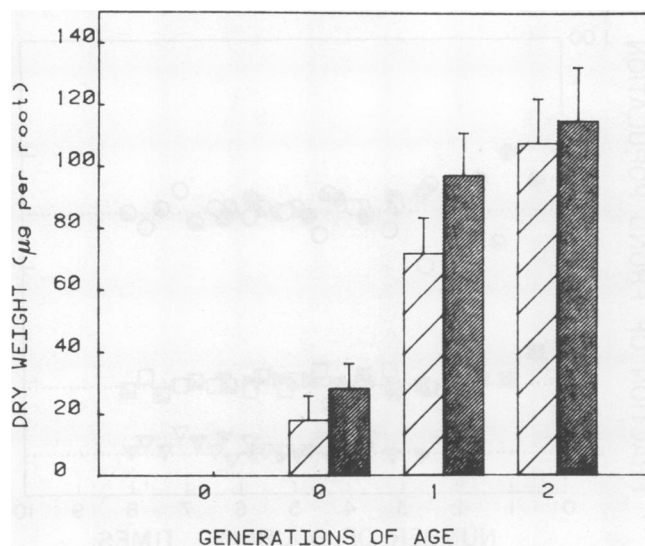

FIG. 4. Root dry weight (μg) as a function of developmental stage and of temperature. Legend as for Figure 1.
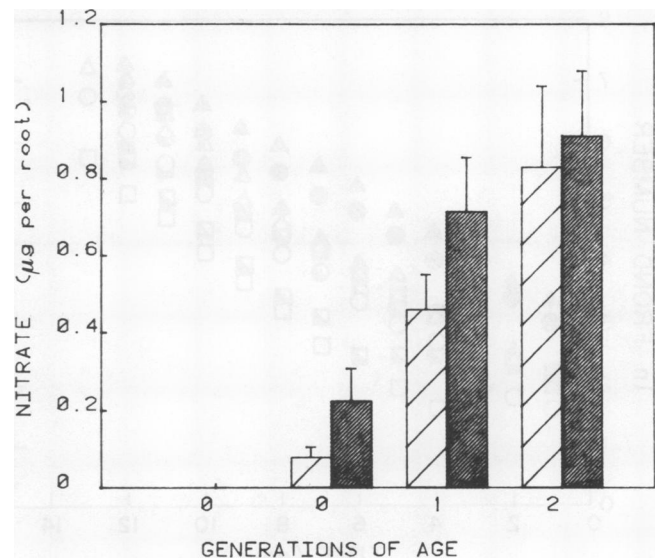

FIG. 6. Root nitrate (μg) as a function of developmental stage and of temperature. Legend as for Figure 1.

tein is markedly affected by incubation temperature and, in plants grown at 23.9 C, appears to be more than 1.5 times the percentage found in plants grown at 18.3 C.

Like the frond, root protein content increases with both generational stage and temperature (Fig. 3; Tables I, II). Protein content per root in young stages is much less than protein per frond. At older stages, the root protein forms a larger fraction of the plant total protein so that roots of grandparent plants contribute more than 25% of the plant protein present at that stage (Fig. 3; cf. Fig. 1). This is because the root continues to grow as the plant ages (Fig. 4; cf. Fig. 2). Protein comprises between 8 and 13% of root dry weight, and this percentage is somewhat greater in plants grown at 23.9 C than in those grown at 18.3 C (Table III).

Nitrate Content of Fronds and Roots. Nitrate content of fronds is greater (1.4- to 6-fold) in plants grown at 23.9 C than in those grown at 18.3 C and does not increase progressively with developmental stage (Fig. 5; Tables I, II). Nitrate content of roots is higher in plants grown at 23.9 C than in those grown at 18.3 C and does increase with generational stage, at least through two

generations of development (Fig. 6; Tables I, II). Adult size, 0 generation plants have slightly more nitrate in roots than in fronds. By the time plants are two generations old, the long roots have 7 to 9 times as much nitrate as the fronds. This preferential partitioning of nitrate in roots and protein in fronds may have agronomically important implications (see "Discussion").

Distribution of Generations within the Population. Since protein and nitrate vary with generational stage (Figs. 1, 3, 5, and 6), estimates of population nitrogen levels require information on the distribution of generations within the population. The number of total fronds in a duckweed population is usually observed to increase exponentially with time if nutrients and space are not limiting (1, 2, 10). Models which would produce this exponential growth are bifurcating patterns of frond production, as indicated in the diagram (Fig. 7). The tracings of Figure 7 (top) and studies reported in the literature show an increase in replication time with sibling number (2, 4, 25) and a rhythmic periodicity associated with handedness (4). In the absence of data on replication times, one can propose the simplest possible bifurcation model, that to a first approximation siblings and progeny are acquired simulta-
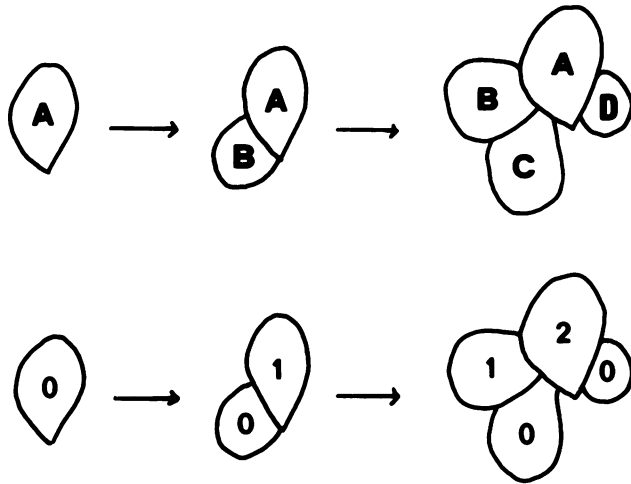
FIG. 7. Bifurcation patterns which produce exponential growth. Upper tracings have been lettered to show fronds in the order in which they arise. In lower tracings, the same fronds have been renumbered to indicate their developmental (generational) stages.
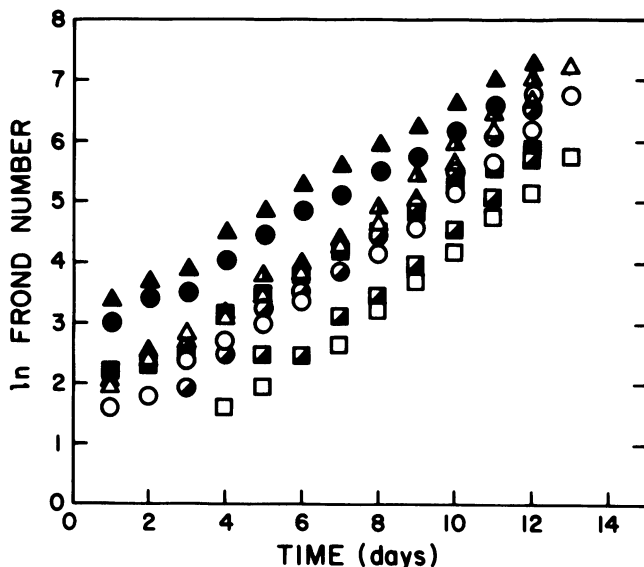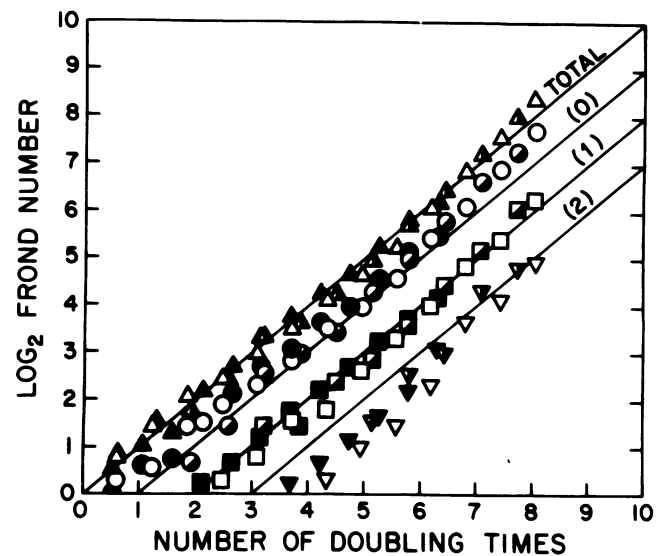

FIG. 9. Dimensionless plot suggested by equation (2) of text reveals relationships among generational stages. Legend as for Figure 8, with additional symbols ($\triangledown$, $\blacktriangledown$) to show number of second generation (grandparents) fronds. Diagonal lines show pattern predicted by equation (2).

FIG. 8. Growth curves for different generations. Symbols indicate generational stages: ($\triangle$, $\blacktriangle$), total fronds; (O, ●), 0 generation (daughter fronds); ($\square$, ■), first generation (parent fronds). Solid symbols, colony incubation at 15 C; half-filled symbols, incubation at 18.3 C; open symbols, incubation temperature at 23.9 C.
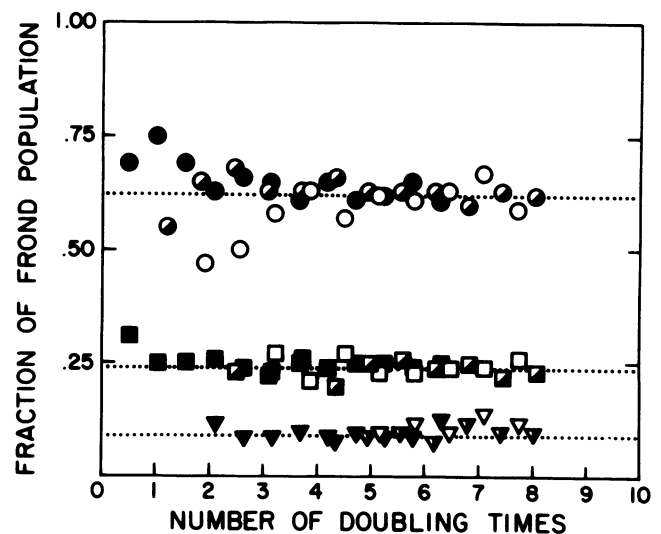

FIG. 10. Distribution of generations in the population. Lines show mean observed values for fraction of total population formed by different generational stages. Legend as for Figure 9.

neously, and test the suitability of the simple model for the population at hand. The simple bifurcation model (Fig. 7, bottom) has the property that

$$N(g) = T \times 2^{-g-1} \qquad (1)$$

where $N(g)$ represents the number of fronds which are g generations old and T is total number of fronds in the population. Another way to express equation (1) is

$$\log_2 N(g) = D - g - 1 \qquad (2)$$

with $\log_2 T = D$ where D represents the number of doubling times.

An implication of equation 2 is that dimensionless plots of the logarithm to the base two of the number of fronds *versus* the number of population doubling times should yield, for the different generations, a set of parallel lines of slope 1, with 1 unit on the x-axis between the lines for successive generations.

When the logarithm of the number of fronds of different stages is plotted against time in the conventional manner, an almost undecipherable pattern results (Fig. 8). The number of individuals in each generation increases exponentially with time, but other relationships are obscured. The relationships among the frond numbers for different generations are made clear in the dimensionless plot suggested by equation 2 (Fig. 9), which reveals that the populations growing at different temperatures develop in a similar manner. At each of three temperatures, the number of total fronds and parent fronds fits the theoretical lines of the simple bifurcation model. There appear to be more daughter (0 generation) and slightly fewer grandparent (second generation) fronds than predicted. For third and subsequent generations (data not shown), the number of fronds found is considerably less than predicted by the model. The inferences from Figure 9 are confirmed by further analysis in Figure 10. The equations imply that, at any time during exponential growth, half of the total frond population have not yet produced offspring, one-quarter are par-

ent fronds, and one-eighth are grandparent fronds. This implication is tested in Figure 10, from which we calculate that the actual fractions are respectively, 0.62, 0.24, and 0.09 for the 0, 1 and 2 generational stages in the population. The remaining 5% of the population are more than two generations old.

While the simple bifurcation model works only to a first approximation, it suggests a valuable dimensionless plot which allows comparison of experiments performed at different temperatures and different initial frond numbers. Tests of the model (Figs. 9 and 10) show that, over a large temperature range, the distribution of generations in the population is constant for more than eight doubling times.

DISCUSSION

Protein content in the Davis *Lemna* fronds is higher than the protein content of the roots, while nitrate content in the roots is much higher than in the fronds (*cf.* Figs. 1, 3, 5, and 6). The enrichment of protein in leaves and nitrate in roots is characteristic of monocotyledons such as barley (9, 12) and is thought to be related to higher levels of nitrate reductase in leaves (19). But it is surprising to find the tissue distinctions in duckweeds, since the fronds float on the nutrient medium and all cells are within a few cell lengths of the nitrate supply (10).

Since nitrate reductase is rapidly substrate-induced in Lemnaceae (6, 7, 14), one might expect the nitrate pool size to remain constant. However, our data (Figs. 1, 3, 5, and 6) indicate that both protein and nitrate accumulate in larger quantities and in higher concentrations at 23.9 C than at 18.3 C. At least two mechanisms could be proposed to explain this. Raising the incubation temperature could cause an increase in the rate of nitrate accumulation over nitrate reduction to produce the larger nitrate and protein pool sizes. There is ample literature evidence to support this possible mechanism. Both nitrate uptake (5, 13, 18) and nitrate reduction (9, 19) have been shown to increase with temperature in monocotyledons. In one case (5), a slightly higher Q_{10} for uptake compared to reduction resulted in an increase in nitrate pool size with temperature.

A second mechanism which could contribute at least part of the increases in nitrate and protein is a possible decrease in size of the amino acid pool which lies in the chemical pathway between nitrate reduction and protein formation. Trewavas (22, 23) recently demonstrated that the amino acid pool is large in *Lemna minor*. When *Lemna* growth is reduced (by incubation without nutrients), protein degradation rates are increased while protein formation rates decline (23). The slower growth rate recorded at 18.3 C might be accompanied by an increase in the pool size of free amino acids at the expense of the nitrate precursor and protein products.

Our study of developmental changes in locally collected duckweeds was inspired by a hope of producing a useful crop from California irrigation return waters as well as a method of removing nitrate from return water. It is well known that wild fowl eat duckweed. Recent feeding tests and investigations (3, 11, 24) have confirmed that duckweed has high concentrations of lysine, arginine, and xanthophylls and makes an acceptable alfalfa substitute in poultry feed. For agronomic purposes, one would wish to maximize protein concentration and minimize nitrate. The distribution of nitrate and protein shown in our work suggests that removing roots would lower nitrate concentrations to safe levels (16) for animal feed. Perhaps roots could be separated from fronds by taking advantage of differences in specific gravity. The fronds, which are rich in protein, could be used as feed, while the nitrate-rich roots could be used for fertilizer.

It is interesting to compare our results with the predictions of a recently published theoretical model of duckweed population growth (4). In an elegant study of *Lemna paucicostata,* Datko *et al.* (4) modeled the distribution of colony types, each of which is

a specific association among fronds of different stages. By including in their model observed replication times for the different frond types and observed separation times for the colonies, they were able to predict the number of colony types as well as the distribution of generations. Their predicted distributions for the number of fronds which have produced (0) and (2) fronds were very close to what we found experimentally for our local duckweed, but our observation that 24% of total fronds occur in the (1) stage differs somewhat from their prediction of 15% for *L. paucicostata.*

The observed constant fraction of each generation in the population, combined with the strong dependence of protein and nitrate on generational stage, implies a simple and reliable quantitative model for protein and nitrate as a function of time after inoculation if plants are grown at constant temperature and light intensity. Based on reports of cycling in frond size, we might expect deviations from present results if experiments are performed over longer (seasonal) time scales (2, 21, 25). The large temperature effects on plant biochemical composition (Table I; Figs. 1, and 5) imply more serious difficulties for modeling under field conditions. Adequate models for protein and nitrate in the plants grown in noncontrolled environments require additional studies of temperature and, also, light intensity (Lehman, unpublished results) effects on tissue chemical content.

In conclusion, we have extrapolated from our laboratory data to make some preliminary estimates of the amount of nitrate which could be absorbed from agricultural irrigation return water by a growing population of duckweeds. Assuming from our protein data that a single plant grown at 18.3 C has absorbed 2.71 μg nitrate-N, then the removal of 10 mg l^{-1} N would require 3.69 $\times$ 10^3 plants l^{-1}. This would represent about 10 doubling times (16 days at 18.3 C) from an inoculum of four fronds per liter or eight doubling times (12.9 days at 18.3 C) after inoculation with 16 fronds per liter. If fronds were grown at 23.9 C, the nitrogen content would be twice as high and the doubling time less; at the higher temperature, it would require only 10.8 days to remove 10 mg nitrate-N from a liter of water. Particularly if colonies are grown in shallow containers to maximize the ratio of plant to medium, duckweeds could be quite effective in removing nitrate from agricultural return water.

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